

Mutagenicity and Metabolism of Vinyl Chloride and Related Compounds

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The various adverse biological effects of vinyl chloride appear to be dependent upon the metabolic conversion of this compound into chemically reactive metabolites. The metabolism of vinyl chloride in mammals and in man, including the formation of monochloroacetic acid and some identified sulfur conjugates is reviewed. Hepatic microsomal mixed function oxidases from rats, mice, and humans were equally effective in transforming vinyl chloride into alkylating agents *in vitro*. Two of the enzyme reaction products, i.e., chloroethylene oxide and 2-chloroacetaldehyde, showed potent genetic activity in microorganisms and Chinese hamster V79 cells. The role of liver microsomal enzymes in the generation of electrophilic mutagenic vinyl chloride metabolites is discussed.

Chlorinated hydrocarbons, such as vinyl chloride (VCM) or vinylidene chloride, are widely used in the production of plastic resins and are present in the environment (1, 2). It is now recognized that VCM is responsible for various adverse biological effects which induce neoplastic and nonneoplastic diseases in man as well as in animals (2) and that these effects seem to be attributable not to VCM itself but to reactive metabolites formed by microsomal mixed-function oxidases. Many chemical carcinogens have been shown to exert a carcinogenic effect following their metabolic activation into electrophilic metabolites which react readily with cellular macromolecules (3).

This paper is a review of results obtained in our laboratory and by others concerning the mutagenicity of VCM and related compounds and the metabolism of some of these compounds.

Mutagenicity

The mutagenicity of VCM has been examined in various strains of *S. typhimurium* in which

the genetic indicator reverts to histidine prototrophy by single-base pair substitutions (G-46, TA1530 and TA1535) and in strains TA1536, TA1537 and TA1538 which revert by base-pair insertions and deletions, or "frameshifts" (8).

Mutagenic responses were observed only in *S. typhimurium* strains which revert by single base-pair substitutions and not in strains TA 1536, TA1537 and TA1538 which are specifically reverted by frameshift mutagens (4, 5). Exposure of *S. typhimurium* strain TA1530 in a soft layer to 20% VCM (v/v) in air in the absence of a mammalian metabolic activation system caused a linear increase in the mutagenic response as a function of incubation time (Fig. 1). This mutagenic effect could be attributed to nonenzymic breakdown products of VCM or to a compound(s) formed by bacterial enzyme systems. However, a much higher mutagenic response was observed in the presence of a fortified liver postmitochondrial supernatant from phenobarbitone-pretreated mice added to the assay systems (Fig. 1). A similar increase in the mutagenic response was noted with 9000 g liver supernatants from phenobarbitone-pretreated or untreated rats (4, 5). In the TA1530 strain, which showed the highest mutagenic response, the number of *his*⁺ revertant

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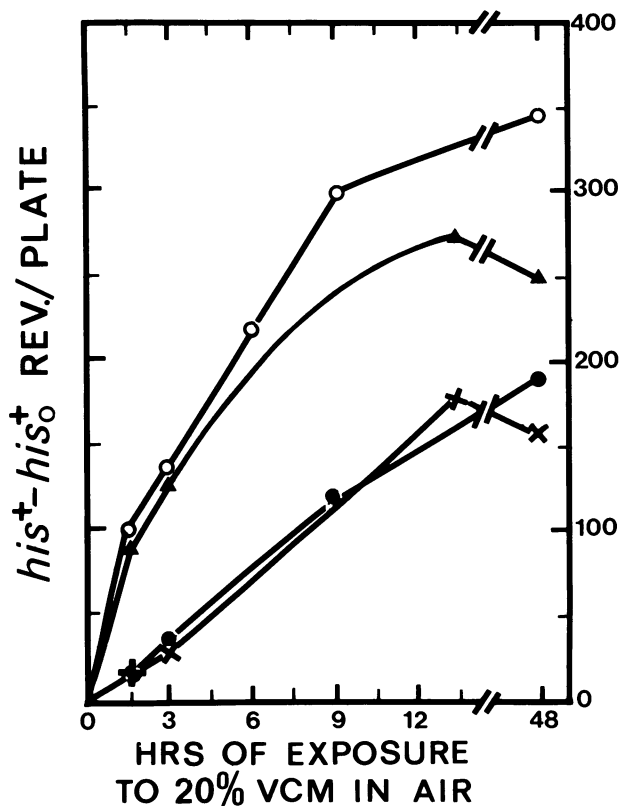


FIGURE 1. Mutagenic response of TA1530 strain after VCM exposure for various lengths of time. Mutagenicity assays (4) were performed with TA1530 and pooled liver fractions from five phenobarbitone-pretreated mice, following exposure of the bacteria to 20% VCM in air at 37°C in the presence of: (o) a 9000g supernatant and a NADPH-generating system; (▲) a 9000g supernatant, a NADPH-generating system, NAD⁺ (4μmole/plate) and alcohol dehydrogenase (5.9 Units/plate); (x) a 100,000g supernatant, a NADPH-generating system, NAD⁺, and alcohol dehydrogenase; (●) KCl and NADP⁺ only.

colonies increased approximately 6, 12 and 28 times over the number of spontaneous mutations after exposure to 0.2, 2.0, and 20% VCM (v/v), respectively, in air for 48 hr (4). Similar results were obtained by Rannug et al. (9) and by McCann et al. (10) using *S. typhimurium* strains TA1535 and TA100.

Loprieno et al. (11) reported a dose-related mutagenic response in the yeast *Saccharomyces pombe* when it was incubated with fortified mouse-liver microsomes and flushed with 50% VCM in air; no mutagenic response was observed when liver microsomes were omitted

from the incubation mixture. In the same incubation system, VCM induced a significant increase in gene conversion frequency in *S. cerevisiae* D₄ strain at the adenine-2 and tryptophan-5 loci (11).

These data strongly support the hypothesis that VCM requires biotransformation by mammalian liver enzymes in order to manifest its genetic effects.

Table 1. Effects of various subcellular tissue fractions on the mutagenic response to VCM.^a

Mouse liver fraction ^b	No. of his ⁺ rev./plate ^c
None (KCl)	96 ± 38
9000 g supernatant (S-9)	310 ± 18
Microsomal fraction	175 ± 20
Cytosol	138 ± 12
Microsomal fraction + cytosol	501 ± 25

^a Data of Malaveille et al. (5).

^b Equivalent to 38 mg of wet tissue/plate; NADPH generating system present.

^c Mutagenicity assays with strain TA1530; 6 hr exposure to 20% VCM in air at 37°C. No. of spontaneous mutations/plate (10 ± 3) subtracted from each value. Mean values ± S.D. from three mutagenicity assays, each utilizing pooled liver tissue from five PB-pretreated mice.

Malaveille et al. (5) investigated the subcellular localization of the VCM activating enzyme(s) by assaying various liver fractions from phenobarbitone-pretreated mice (Table 1) for their capacity to form a VCM metabolite(s) mutagenic for *Salmonella typhimurium* TA1530 strain. In the presence of a NADPH-generating system (NADP⁺ and glucose-6-phosphate), the highest enzyme activity was found to be localized in either the 9000 g supernatant or in the recombined microsomal and soluble protein fraction. Purified liver microsomal fractions plus VCM induced a lower mutagenic response, and the omission of the NADPH-generating system from the assays resulted in a number of his⁺ revertants similar to that in control assays containing KCl only. Soluble liver proteins (100,000 g supernatant) did not increase the mutagenic response to VCM. The addition of alcohol dehydrogenase and NADP⁺ to either the fortified postmitochondrial fraction or to the 100,000g

liver supernatant did not increase the mutagenic response to VCM (Fig. 1).

The involvement of microsomal enzymes in VCM metabolism was further demonstrated by the changes in the tissue-mediated mutagenicity of VCM resulting from administration of modulators of microsomal mixed-function oxidases to animals. The addition of postmitochondrial liver fractions from phenobarbitone-pretreated rats or mice resulted in an increased mutagenic response *in vitro* when compared with that in the presence of fractions from untreated animals (4, 5). Incubation media containing 9000 g liver fractions from rats pretreated with aminoacetonitrile, pregnenolone-16 α -carbonitrile or bis(diethylthiocarbamoyl)disulfide (disulfiram) reduced the liver enzyme-mediated mutagenic effect of VCM in *S. typhimurium* TA1530 (2). The reduction in the mutagenic effect of VCM observed when disulfiram was added directly to assay systems containing 9000 g liver fractions from mice can be explained either as a direct effect on microsomal enzyme activity or by the presence of nucleophilic SH-groups formed from cleavage of disulfiram, which could compete for the binding of the mutagenic metabolites to bacterial DNA.

Bartsch et al. (4) and Malaveille et al. (5) compared the mutagenic effects in *S. typhimurium* TA1530 strain of various putative or identified vinyl chloride metabolites. VCM (monochloroethylene) is oxidized *in vitro* by microsomal mixed-function oxidases to chloroethylene oxide, which rearranges spontaneously to chloroacetaldehyde (12). Hefner et al. (13) have proposed that 2-chloroethanol is an intermediate in the metabolism of vinyl chloride or VCM, and monochloroacetic acid is a VCM metabolite which has been identified in the urine of rats and humans (14, and see next section). Three of the four VCM derivatives tested in *S. typhimurium* TA1530 strain exerted a mutagenic effect; monochloroacetic acid had only a toxic effect. Chloroethylene oxide elicited the strongest mutagenic response but had a relatively low toxicity when compared to that of 2-chloroacetaldehyde, which was also found to be mutagenic. 2-Chloroethanol increased the number of *his*⁺ revertants to 10 times the spontaneous mutation rate, only in the presence of a post-mitochondrial mouse liver fraction. The postmitochon-

drial mouse liver fraction decreased the mutagenic effect of 2-chloroacetaldehyde.

Similarly, in *S. typhimurium* TA100 strain, only 2-chloroacetaldehyde and 2-chloroethanol, and not monochloroacetic acid, were found to be mutagenic (10). In a more detailed study, Rannug et al. (15) also examined the mutagenicity of these four VCM metabolites in *S. typhimurium* TA1535; he reached the conclusions that chloroacetaldehyde was less effective by a factor of 1000, than chloroethylene oxide in inducing point mutation in this strain and that 2-chloroethanol is unlikely to be responsible for the mutagenicity of VCM in this test system. Monochloroacetic acid was not mutagenic at any concentration tested.

Similar results were obtained by Huberman et al. (6) in Chinese hamster V-79-4 cells: a dose-dependent induction of 8-azaguanine- or ouabain-resistant mutants was observed with chloroethylene oxide, whereas 2-chloroacetaldehyde induced only 8-azaguanine-resistant mutants and resulted in a high cytotoxicity. 2-Chloroethanol and monochloroacetic acid were inactive even at concentrations up to 100 times as high.

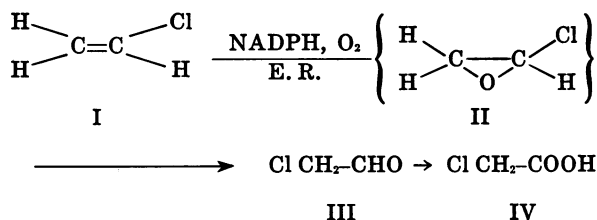
Metabolism

The pharmacokinetics and tissue distribution of ¹⁴C-labeled VCM in rats exposed by various routes to different concentrations was reported by Watanabe et al. (16, 17). For animals exposed to 1000 ppm ¹⁴C-VCM for 6 hr, 56% of the radioactivity was excreted in urine, 12% was expired as VCM, 12% was expired as ¹⁴CO₂, and 4% was found in the feces within 72 hr. For animals administered 10 ppm within 6 hr, 68% of the ¹⁴C radioactivity was recovered from the urine and 2% in expired ¹⁴C-VCM. With single oral or intraperitoneal administration, low doses of VCM resulted in higher excretion of radioactivity in the urine and lower exhalation as ¹⁴C-VCM, while high dose levels resulted in the opposite pattern of elimination (17, 18). Following a single intravenous injection of 250 μ g/kg ¹⁴C-VCM, 80% of the radioactivity was detected within a few minutes in the exhaled air as unchanged VCM (18). Of the various tissues examined, the liver and skin retained the highest levels of radioactivity (16, 17).

These data indicate that at low doses VCM is metabolized to polar metabolites which are

excreted in the urine. Two of the three major urinary metabolites detected have been identified as *N*-acetyl-*S*-(2-hydroxyethyl)-cysteine and thiodiglycolic acid, suggesting that the primary VCM metabolites are excreted in the urine as glutathione or cysteine conjugates. No qualitative or quantitative differences among these metabolites were observed in rats with the various doses and exposures examined (19).

The occurrence of these urinary metabolites substantiates the evidence provided previously (2) that VCM (I) is metabolized by microsomal mixed-function oxidases to a primary reactive metabolite, chloroethylene oxide (II), which rearranges to chloroacetaldehyde (III); a further oxidation leads to monochloroacetic acid (IV).



The metabolic activation of VCM to a mutagenic metabolite(s) by liver microsomal systems from various animal species as well as from humans has been shown to be dependent on the presence of the cofactors necessary for mixed-function oxidase reactions (NADPH-generating system and oxygen) (4, 9, 11). With ^{14}C -VCM, it has been demonstrated that liver microsomes in the presence of the necessary cofactors are essential for the binding of a VCM metabolite(s) to cellular macromolecules (20, 21).

Administration of inducers and inhibitors of drug metabolizing enzymes to animals has been shown to modify microsome-mediated binding to nucleic acids and the mutagenicity of VCM *in vitro* (2, 22). The potent mutagenic activity of chloroethylene oxide, when compared to that of the other VCM metabolites examined (5, 15), strongly suggests that this alkylating metabolite is responsible for the adverse biological effects of VCM. In an aqueous solution at pH 7.4 and 37°C, chloroethylene oxide has a half-life of 1.6 min, and its strong alkylating activity was demonstrated by its reaction with 4-(*p*-nitrobenzyl)pyridine. Under the same experi-

mental conditions, 2-chloroacetaldehyde showed no reactivity (7).

As chloroethylene oxide readily rearranges to 2-chloroacetaldehyde (23), this latter, volatile compound has been detected after the metabolic conversion of VCM by liver microsomal mixed-function oxidases and oxygen (24). The primary reactive metabolite, chloroethylene oxide, could be detected *in vitro* when a mixture of VCM and oxygen (1:1, v/v) was passed through a medium containing liver microsomes from phenobarbitone-pretreated mice and a NADPH generating system. A volatile metabolite was trapped with 4-(*p*-nitrobenzyl)pyridine in ethylene glycol, and its absorption spectrum was shown to be identical with that obtained from chloroethylene oxide (Fig. 2),

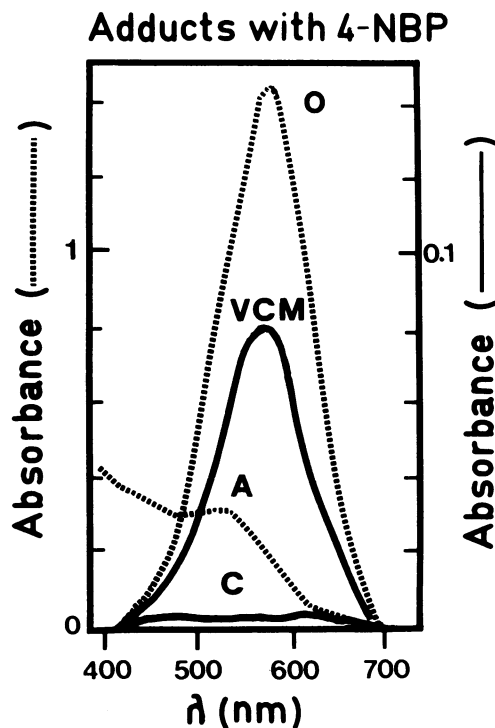


FIGURE 2. Absorption spectra of VCM, metabolites with 4-(*p*-nitrobenzyl)pyridine: absorption spectra recorded after reaction of 4-(*p*-nitrobenzyl)pyridine with (O) chloroethylene oxide or (A) with 2-chloroacetaldehyde; spectrum of VCM recorded by passing a mixture of VCM/oxygen through a medium consisting of liver microsomes from phenobarbitone-pretreated mice and a NADPH-generating system and reacting the volatile metabolite with 4-(*p*-nitrobenzyl)pyridine in ethylene glycol; (C) control in which NADP⁺ and glucose-6-phosphate were omitted (7).

Table 2. Comparative mutagenicities of olefinic compounds.^a

Compound	<i>his</i> + revertant colonies/ μ mole/hr/plate ^b				Carcinogenicity
	TA1530		TA100		
	+ cofactors ^c	– cofactors	+ cofactors ^c	– cofactors	
Vinyl acetate	0	0	0	0	—
Vinyl chloride	3.7	1.2	5.6	1.5	+
Vinylidene chloride	9.5	0.2	14.6	0.7	?
2–Chlorobutadiene	34.5	12.5	51.2	9	?

^a Data of Bartsch et al. (30,31).

^b From linear dose- and time-dependent response curves in the presence of mouse liver 9000g supernatant; spontaneous mutations subtracted.

^c NADP⁺, glucose-6-phosphate.

whereas a different spectrum was observed for 2-chloroacetaldehyde. In the absence of a NADPH generating system, no such product was formed from VCM.

In another series of experiments, Barbin et al. (7) showed that chloroethylene oxide or 2-chloroacetaldehyde react covalently with adenosine *in vitro*. After chromatography on Sephadex G-10, some of the reaction products obtained from VCM and adenosine in the presence of a microsomal system were eluted at positions similar to those found with chloroethylene oxide or 2-chloroacetaldehyde. 2-Chloroacetaldehyde reacts *in vitro* with adenosine and cytidine to give fluorescent products (25).

Mutagenicity tests similar to those used in the studies on VCM were used to investigate some related olefinic compounds which are also used in the plastic and rubber industries (2, 27). Table 2 lists the relative mutagenic strengths (number of *his*⁺ revertant colonies/ μ mole of substrate/hr of exposure/plate) induced both directly and when mediated by mouse liver enzyme of VCM, vinylidene chloride (1,1-dichloroethylene) and 2-chloro-1,3-butadiene (chloroprene) in *S. typhimurium* strains TA-1530 and TA100. 2-Chlorobutadiene exhibited the highest microsome-mediated mutagenic effect and was also mutagenic in the absence of the mouse liver microsomal fraction. With vinylidene chloride, no mutagenic effect was observed when the microsomal activation system was omitted from the incubation mixture. Vinyl acetate, a noncarcinogenic compound (28), was not mutagenic in this experimental system. Data on the carcinogenicity of vinyli-

dene chloride and 2-chlorobutadiene in animals are not yet available, but long-term carcinogenicity tests are under way in the IARC laboratory and at other institutes.

Since epoxides are now recognized to be obligatory intermediates in the metabolism of many olefinic compounds by hepatic microsomal mixed-function oxidases (29), it can be postulated that they may be the primary reactive metabolites of these compounds. This is strongly suggested to be true for VCM. Microsomal fractions of human liver biopsy specimens have also been shown to metabolize these halogenated olefins into mutagenic and electrophilic metabolites (30).

Conclusions

The experimental data available at present strongly support the concept that the biological effects of VCM are related to its conversion by microsomal enzymes into chemically reactive alkylating agents which can bind covalently to various cellular macromolecules. A similar mechanism of action has been implicated for other classes of carcinogens.

Based on mutagenicity studies with various genetic indicators, the data strongly suggest that chloroethylene oxide is the VCM metabolite principally responsible for the various adverse biological effects of the parent compound. The role of chloroacetaldehyde in VCM carcinogenesis needs to be studied in long-term carcinogenicity tests, but its diacetal derivative has been reported to be noncarcinogenic (26).

The increasing evidence of an empirical correlation between the carcinogenic and mutagenic effects of chemicals, together with the present findings, emphasize the need for a careful evaluation of the carcinogenic potential of those chemicals structurally related to VCM.

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